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**Acute treatment with bone marrow-derived mononuclear cells attenuates the organ injury/dysfunction induced by hemorrhagic shock in the rat**

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## ABSTRACT

**Background:** Recent evidence suggests that stem cells such as bone marrow-derived mononuclear cells (BMMNC) have both regenerative and paracrine properties. Here we investigate the effects of BMMNC on the organ injury/dysfunction induced by hemorrhagic shock.

**Methodology and principal findings:** Thirty-seven anesthetised male Wistar rats were subjected to hemorrhage by reducing mean arterial pressure to  $35 \pm 5$  mmHg for 90 min, followed by resuscitation with 20 ml/kg Ringer's lactate administered over 10 min and 50% of the shed blood over 50 min. Rats were sacrificed 4 h after the onset of resuscitation. BMMNC were freshly isolated from rat tibias and femurs using Percoll density gradient centrifugation and BMMNC ( $1 \times 10^7$  cells per rat in 1 ml/kg PBS, i.v.) were administered on resuscitation. Hemorrhagic shock resulted in significant organ injury/dysfunction (renal, hepatic, neuromuscular) and inflammation (hepatic, lung). In rats subjected to hemorrhagic shock administration of BMMNC significantly attenuated (i) organ injury/dysfunction (renal, hepatic, neuromuscular) and inflammation (hepatic, lung), (ii) increased the phosphorylation of Akt and glycogen synthase kinase-3 $\beta$ , (iii) attenuated the activation of nuclear factor- $\kappa$ B, (iv) attenuated the increase in extracellular signal-regulated kinase-1/2 phosphorylation and (v) attenuated the increase in expression of ICAM-1.

**Conclusions:** Our findings suggest that administration of BMMNC protects against multiple organ injury/dysfunction caused by severe hemorrhagic shock by a mechanism that may involve activation of Akt and glycogen synthase kinase-3 $\beta$  and the inhibition of nuclear factor- $\kappa$ B.

## INTRODUCTION

Trauma and the associated blood loss is a leading cause of death in under 45 year olds in the United States of America [1]. Extreme blood loss or hemorrhage causes hypoperfusion of tissues and the consequent cellular hypoxia and hypoglycaemia metabolically damages tissues leading to impairment of organ function [2]. In order to prevent complete exsanguination, fluid resuscitation is used to restore circulating volume until suitable cross-matched blood transfusions can be administered [3]. However, many resuscitation fluids such as crystalloids (e.g. Ringer's lactate) are known contribute to tissue injury [4]. This is due to the return of oxygen to ischemic tissues; production of reactive oxygen species and activation of immune cells induces the systemic inflammatory response syndrome (SIRS) which can contribute to apoptosis and tissue necrosis leading to further organ injury [5]. In patients with trauma, failure of more than four organs is linked to certain mortality therefore highlighting the need for interventions that may reduce or prevent the deterioration in organ injury and function [6].

Recent evidence suggests that stem cells such as bone marrow-derived mononuclear cells (BMMNC) have both regenerative and paracrine properties [7]. Stem cells secrete anti-inflammatory proteins *in vivo* which reduce the injury caused by ischemia and reperfusion [8]. Takahashi et al. (2006) [9] demonstrated that intra-myocardial administration of BMMNC significantly reduced the infarct size in a rat model of myocardial infarction and that BMMNC secreted VEGF, IL-1 $\beta$ ,  $\beta$ FGF, PDGF, IGF-1, and TGF- $\beta$  *in vitro* supporting the paracrine role of stem cells. BMMNC also reduce the (i) tissue injury and dysfunction caused by regional myocardial [8]

and cerebral ischemia-reperfusion [10]; (ii) attenuate the lung dysfunction induced by lipopolysacchride [11] and (iii) prevent the formation of granulomas in silica-induced lung injury [12].

The mechanism(s) by which stem cells may produce these protective effects is still unknown. There is evidence that the paracrine mediators released by stem cells may act on cell survival pathways such as the PI3K/Akt pathway [8,13] and MAPK pathways [14], to downregulate the expression of pro-inflammatory and pro-apoptotic proteins and upregulate the expression of anti-inflammatory and anti-apoptotic proteins. Understanding the mechanism(s) by which stem cells are protective could lead to the development of characterised interventions that can specifically act on these mechanisms without the need for cellular infusions [7].

This study investigates the effects BMMNC treatment could have on the organ injury/dysfunction induced by hemorrhagic shock (HS). HS causes ischemia of many organs and tissues and the subsequent resuscitation frequently results in a 'reperfusion-type' injury. In our study, BMMNC were administered by intravenous injection on resuscitation. Having discovered that BMMNC given on resuscitation reduce organ injury/dysfunction, we have designed a number of further studies to gain a better understanding of their mechanism of action. These include the effects of BMMNC on the expression and activation of various cellular signalling pathways [in particular phosphorylation of Akt on Ser<sup>473</sup>, phosphorylation of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) on Ser<sup>9</sup>, activation of nuclear factor (NF)- $\kappa$ B (measured as nuclear translocation of p65), activation of p38 MAPK, activation of extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK) 1/2].

## RESULTS

***Effect of BMMNC administration on the circulatory failure caused by hemorrhagic shock.*** When compared to sham-operated rats, HS-rats treated with vehicle demonstrated a significant reduction in mean arterial pressure (MAP) during the resuscitation period ( $P < 0.05$ , Fig 1). Administration of BMMNC was sufficient to increase MAP during resuscitation so that it was similar to that of sham-operated rats ( $P < 0.05$ , Fig 1), although this declined towards the end of the experiment. Administration of BMMNC in sham-operated rats had no significant effect on MAP when compared to vehicle treated sham-operated rats ( $P > 0.05$ , Fig 1).

***Effect of BMMNC administration on the organ injury, dysfunction and inflammation caused by hemorrhagic shock.*** When compared to sham-operated rats treated with vehicle, HS-rats treated with vehicle developed significant increases in serum urea ( $P < 0.05$ , Fig 2A), creatinine ( $P < 0.05$ , Fig 2B), AST ( $P < 0.05$ , Fig 2C), ALT ( $P < 0.05$ , Fig 2D) and creatine kinase ( $P < 0.05$ , Fig 2E) indicating the development of renal dysfunction, liver injury and skeletal-muscle injury. Treatment of HS-rats with BMMNC significantly attenuated the rises in serum urea ( $P < 0.05$ , Fig 2A), creatinine ( $P < 0.05$ , Fig 2B), AST ( $P < 0.05$ , Fig 2C), ALT ( $P < 0.05$ , Fig 2D) and creatine kinase ( $P < 0.05$ , Fig 2E). There was no significant difference in levels of serum urea ( $P > 0.05$ , Fig 2A), creatinine ( $P > 0.05$ , Fig 2B), AST ( $P > 0.05$ , Fig 2C), ALT ( $P > 0.05$ , Fig 2D) and creatine kinase ( $P > 0.05$ , Fig 2E) between sham-operated rats treated with either vehicle or BMMNC.

1 Myeloperoxidase (MPO) activity was determined in order to estimate the  
2 degree of immune cell infiltration in both the liver and lung. When compared to  
3 sham-operated rats, rats subjected to HS and treated with vehicle had significantly  
4 higher MPO activity ( $P<0.05$ , figure 3A and 3B). BMMNC treatment significantly  
5 reduced the MPO activity in both organs therefore reducing the infiltration of immune  
6 cells into the lung and liver ( $P<0.05$ , figure 3A and 3B).  
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17 ***Effect of BMMNC on the phosphorylation of Akt and GSK-3 $\beta$  in the livers***  
18 ***and lungs of rats that underwent hemorrhage and resuscitation.*** In order to gain  
19 a better insight into the potential mechanism(s) underlying the observed beneficial  
20 effects of BMMNC we investigated the effects of these BMMNC on cell signalling  
21 pathways known to confer tissue protection or to inhibit inflammation (in liver and  
22 lung). When compared to sham-operated rats, HS-rats treated with vehicle  
23 developed significant decreases in the phosphorylation of Akt on Ser<sup>473</sup> and GSK-3 $\beta$   
24 on Ser<sup>9</sup> in liver and lung ( $P<0.05$ , Fig 4A-D). Treatment of HS-rats with BMMNC  
25 attenuated the decline in the phosphorylation of Akt and GSK-3 $\beta$  caused by  
26 hemorrhage-resuscitation in both liver and lung ( $P<0.05$ , Fig 4A-D).  
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44 ***Effect of BMMNC on the nuclear translocation of the p65 NF- $\kappa$ B subunit***  
45 ***in the livers and lungs of rats that underwent hemorrhage and resuscitation.***  
46 When compared to sham-operated rats, HS-rats treated with vehicle developed  
47 significant increases in the nuclear translocation of the p65 NF- $\kappa$ B subunit in both  
48 liver and lung indicating the activation of NF- $\kappa$ B ( $P<0.05$ , Fig 5A and 5B). Treatment  
49 of HS-rats with BMMNC resulted in a significant reduction in nuclear translocation of  
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1 p65 and, hence, the activation of NF- $\kappa$ B in both liver and lung (P<0.05, Fig 5A and  
2 5B).  
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7 ***Effect of BMMNC on the phosphorylation of ERK1/2 MAPK in the livers***  
8 ***and lungs of rats that underwent hemorrhage and resuscitation.*** When  
9 compared to sham-operated rats, HS-rats treated with vehicle developed a  
10 significant increase in the phosphorylation of ERK1/2 in the liver and lung (P<0.05,  
11 Fig 6A and 6B). Treatment of HS-rats with BMMNC attenuated the increase in ERK  
12 phosphorylation caused by hemorrhage and resuscitation in both organs (P<0.05,  
13 Fig 6A and 6B).  
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26 ***Effect of BMMNC on the expression of ICAM-1 in the livers and lungs of***  
27 ***rats that underwent hemorrhage and resuscitation.*** When compared to sham-  
28 operated rats, HS-rats treated with vehicle developed significant increase in the  
29 expression of ICAM-1 in the liver and lung (P<0.05, Fig 7A and 7B). Treatment of  
30 HS-rats with BMMNC attenuated the increase in ICAM-1 expression caused by  
31 hemorrhage and resuscitation in both the liver and lung (P<0.05, Fig 7A and 7B).  
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## DISCUSSION

Recent evidence indicates that stem cell therapy with e.g. BMMNC, can protect organs and tissues against ischemia and reperfusion injury [15-17]. We have previously demonstrated that acute administration of BMMNC ( $1 \times 10^7$  cells i.v.) on resuscitation significantly reduced the infarct size in a rat model of myocardial reperfusion injury; and this protection was attributed to the activation of the PI3K/Akt pathway [8]. We and others have speculated that stem cells are able to secrete paracrine factors such as cytokines, chemokines and growth factors which act on host tissues to activate cell survival pathways [7]. We report here that acute treatment with BMMNC ( $1 \times 10^7$  cells: c-Kit<sup>+</sup>, CD34<sup>+</sup>, CD45<sup>+</sup>, CD133<sup>+</sup>) attenuated the renal dysfunction, liver injury and neuromuscular injury caused by severe hemorrhage and resuscitation in the anesthetized rat. Treatment with BMMNC also attenuated the liver and lung inflammation induced by hemorrhagic shock demonstrated as a significant reduction in the immune cell infiltration. Multiple organ failure is observed in septic patients and those who have suffered hemorrhage and resuscitation, and is often fatal. The inflammatory component of resuscitation injury causes neutrophil-dependent cell death in tissues across the body which has detrimental effects on an organ's ability to function [2,18]. Failure of more than four organs is associated with certain mortality [6] therefore the development of interventions such as stem cell therapy, are important.

What, then, is the mechanism(s) by which BMMNC exert these beneficial effects? The effects of BMMNC on circulatory failure caused by hemorrhage-resuscitation were transient and are, therefore, unlikely to contribute to the beneficial

1 effects observed. Many interventions that exert protective effects against ischemia-  
2 reperfusion injury including BMMNC do so via activation of cell survival pathways  
3 and inhibition of anti-inflammatory pathways [19,20]. Akt is a member of the  
4 phosphoinositide 3-kinase signal transduction enzyme family, which regulate cellular  
5 activation, inflammatory responses, chemotaxis, and apoptosis [21]. When  
6 phosphorylated by its upstream regulator, phosphoinositide-dependent kinase, Akt  
7 modulates cell survival and growth [21]. We report here that hemorrhage and  
8 resuscitation results in a significant reduction in the phosphorylation of Akt in both  
9 the liver and lung. A reduction in the activation of this important survival pathway will  
10 make organs more susceptible to injury and inflammation [22,23]. Most notably,  
11 BMMNC treatment restored the degree of Akt phosphorylation to the level seen in  
12 sham-operated animals; this effect of BMMNC is similar to those already reported in  
13 hearts subjected to ischemia-reperfusion [8].  
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34 GSK-3 $\beta$  is a serine-threonine kinase that was originally recognized as a  
35 kinase that phosphorylates glycogen synthase. In contrast to most other kinases,  
36 GSK-3 $\beta$  is active in a resting cell state; however, it is inactivated by phosphorylation  
37 of Ser<sup>9</sup>. GSK-3 $\beta$  is regulated by multiple signalling pathways including the Akt  
38 pathway, which inactivates it by causing Ser<sup>9</sup> phosphorylation [24,25]. Consistent  
39 with decline in the phosphorylation/activation of Akt reported here, hemorrhage and  
40 resuscitation also caused a significant decline in the phosphorylation of GSK-3 $\beta$  on  
41 Ser<sup>9</sup>. This indicates an excessive activation of GSK-3 $\beta$  which would drive both  
42 inflammation [26] and tissue-injury [27]. Similar to the above reported effects on Akt  
43 phosphorylation, BMMNC treatment restored the degree of Ser<sup>9</sup> phosphorylation on  
44 GSK-3 $\beta$  to the levels seen in sham-operated animals. An increase in Ser<sup>9</sup>  
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phosphorylation results in inhibition of this kinase and inhibitors of GSK-3 $\beta$  exert potent anti-inflammatory [26,28] and anti-ischemic effects in a number of organs [27,29,30].

Downstream of GSK-3 $\beta$ , several studies have now reported an association between GSK-3 $\beta$  and NF- $\kappa$ B activity *in vitro* [31,32] and *in vivo* [26,33]. NF- $\kappa$ B is a transcriptional factor that plays an important role in regulating the transcription of a number of genes, especially those involved in producing mediators involved in local and systemic inflammation, such as cytokines, chemokines, cell adhesion molecules, apoptotic factors, and other mediators [34]. Treatment of tumor necrosis factor- $\alpha$  stimulated hepatocytes with a specific GSK-3 $\beta$  inhibitor resulted in a decrease of the NF- $\kappa$ B-dependent gene transcription [35]. This study also indicated four potential phosphorylation sites for GSK-3 $\beta$  on the NF- $\kappa$ B subunit p65. Most notably, pretreatment with a number of chemically distinct inhibitors of GSK-3 $\beta$  attenuates organ injury and dysfunction caused by hemorrhage and resuscitation and endotoxemia [26,28]. This protective effect was associated with inhibition of the activation of NF- $\kappa$ B and NF- $\kappa$ B-dependent proinflammatory genes, along with a reduced phosphorylation of Ser<sup>536</sup> on the NF- $\kappa$ B p65 subunit. In addition, GSK-3 $\beta$  may also inhibit the activation of NF- $\kappa$ B by phosphorylating and degrading I $\kappa$ B $\alpha$ , which is required to prevent NF- $\kappa$ B translocation [32]. We report here that hemorrhage and resuscitation results in a significant increase in the activation of NF- $\kappa$ B (measured here as nuclear translocation of p65); which was attenuated with administration of BMMNC. All of the above findings support the view that BMMNC

1 treatment restores the activation of Akt resulting in inhibition of GSK-3 $\beta$  (after  
2 phosphorylation on Ser<sup>9</sup>) and inhibition of the activation of NF- $\kappa$ B.  
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7 Inhibition of NF- $\kappa$ B results in an anti-inflammatory phenotype therefore  
8 reducing the expression of pro-inflammatory proteins such as cytokines, chemokines  
9 and adhesion molecules. In this study BMMNC treatment significantly attenuated the  
10 increase in ICAM-1 expression induced by hemorrhagic shock thus confirming that  
11 the changes in the above signalling pathways lead to an improvement in the  
12 inflammatory state induced by hemorrhage and resuscitation.  
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24 Like ischemia, severe hemorrhage and resuscitation results in the activation  
25 of ERK1/2 and drugs that prevent the activation of ERK1/2 in hemorrhagic shock  
26 exert beneficial effects [36]. We report here that the activation of both ERK1/2  
27 caused by hemorrhage and resuscitation is attenuated by BMMNC treatment. It  
28 should be noted that hemorrhage and resuscitation had no effect on the  
29 phosphorylation states of eNOS, JNK and p38 MAPK (data not shown). Similarly,  
30 administration of BMMNC on resuscitation had no effect on the phosphorylation of  
31 these proteins.  
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47 It should be noted that BMMNC consist of a mixture of different cell types so  
48 the benefit observed in this study may or may not be attributable to a particular cell  
49 type. For example, mononuclear cells from the bone marrow include mesenchymal  
50 stem cells [37] and endothelial progenitor cells [38] both of which have demonstrated  
51 efficacy in animal models of ischemia/reperfusion injuries. The mixture of cell types  
52 we have used here may be more beneficial than using single homogenous  
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populations as the protective effects of many different types of cells can act together. However, this can only be evaluated by comparing the effects of individual cell types versus a mixture.

There are, however, limitations to our study as the model of hemorrhagic shock used here is acute and therefore the data obtained cannot be extrapolated to predict the effects of BMMNC on the survival following haemorrhagic shock. Although this would be advantageous current UK legislation states that such experiments are not possible.

## CONCLUSION

We have discovered that the acute administration of BMMNC on resuscitation attenuates the multiple organ injury and dysfunction in rats subjected to severe hemorrhage and resuscitation. We have previously reported that BMMNC therapy was effective in reducing the infarct size in a rat model of myocardial infarction via activation of the Akt-survival pathway resulting in inhibition of GSK-3 $\beta$  and NF- $\kappa$ B. In addition, proteomic analysis demonstrated an increase in the expression of a number of proteins associated with cardioprotection [8]. In this study, treatment of rats with BMMNC restored the phosphorylation and, hence, activation of Akt, which in turn resulted in inhibition of GSK-3 $\beta$  (secondary to phosphorylation on Ser<sup>9</sup>) and inhibition of the activation of NF- $\kappa$ B. We propose that both effects culminated in a significant reduction in tissue injury and inflammation. There is now very good evidence that therapeutic strategies which enhance the activation of Akt and reduce the activation of GSK-3 $\beta$  enhance the resistance of organs to noxious stimuli (including ischemia) and reduce inflammation via inhibition of NF- $\kappa$ B [32]. In addition, BMMNC treatment attenuated the hemorrhage/resuscitation-induced activation of ERK1/2, which is known to contribute to the development of organ injury/inflammation in hemorrhagic shock [36]. We propose that all of the above signalling events initiated by BMMNC treatment contribute to the beneficial effects in hemorrhagic shock and complement the effects of the paracrine mediators secreted by the BMMNC. Whether this is indeed the mechanism by which stem cells can exert protection, this finding contributes to the evidence that stem cell therapy may be therapeutically viable.

## METHODS

**Ethics Statement.** This study was approved by the ethics committee of Queen Mary University of London and the UK Home Office (PPL: 70/6525) and all procedures were performed strictly under the United Kingdom Animals (Scientific Procedures) Act 1986.

**Surgical Procedure and quantification of organ injury/dysfunction.** This study was carried out on 37 male Wistar rats (Charles River Ltd, Margate, UK) weighing  $295 \pm 7$  g receiving a standard diet and water *ad libitum*. All data from rats that had died during the experiment were excluded from data analysis; a total of 2 animals were excluded from the analysis both of which were from the untreated HS group. Hence the numbers (n) presented represent the 'survivors' of the entire experimental protocol.

Rats were anaesthetised using sodium thiopentone (120 mg/kg i.p. maintained using ~10 mg/kg i.v.) and cannulation of the trachea, carotid artery and jugular vein was performed. Blood was withdrawn via a cannula inserted in the right carotid artery in order to achieve a fall in mean arterial pressure (MAP) to  $35 \pm 5$  mmHg within 10 min. From this point onwards, MAP was maintained at  $35 \pm 5$  mmHg for a period of 90 min either by further withdrawal of blood during the compensation phase or administration of Ringer's Lactate i.v. during the decompensation phase. The average volume of blood withdrawn during haemorrhage was  $9.8 \pm 0.26$  ml (n=23, across all groups). At 90 min after initiation of hemorrhage, resuscitation was performed with 20 ml/kg Ringer's Lactate over a



period of 10 min and then half the shed blood mixed with 100 iu/ml heparinised saline over a period of 50 min. At the end of 1 h resuscitation, an i.v. infusion of Ringer's Lactate (1.5 ml/kg/h) was started as fluid replacement and maintained throughout the experiment for a further 3 h at which point 1.2 mL blood samples were collected via the carotid artery into S/1.3 tubes containing serum gel (Sarstedt, Numbrecht, Germany), after which the heart was removed to terminate the experiment. The blood was centrifuged (9000 g for 3 minutes) to separate serum from which creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipase and creatine kinase were measured within 24 hours (IDEXX Laboratories Ltd., West Yorkshire, UK). Additionally, lung and liver samples were taken and stored at -80°C for further analysis.

***Isolation of Bone Marrow Derived Mononuclear Cells.*** Bone marrow was freshly isolated from femurs and tibias of male Wistar rats, mononuclear cells were isolated by Percoll density gradient centrifugation (Histopaque-1077, Sigma U.K.), as previously described [8] and were resuspended in PBS. BMMNC were characterized by flow cytometry using monoclonal antibodies for c-kit (Santa Cruz, sc-5535, USA), CD34 (Santa Cruz, sc-9095, USA), CD45 (BD, 554875, USA) and CD133 (Santa Cruz, sc-30219, USA). BMMNC were characterised to be c-kit<sup>+</sup> (7 ± 1%, n=10), CD34<sup>+</sup> (7 ± 1%, n=10), CD45<sup>+</sup> (54 ± 6%, n=10) and CD133<sup>+</sup> (15 ± 1%, n=10). BMMNC from one donor rat was used to treat upto three rats that were either sham-operated or subjected to HS.

***Experimental Design.*** Rats were randomly allocated into the following groups: (i) ***sham + PBS*** (n=10), (ii) ***sham + BMMNC*** (n=4), (iii) ***HS + PBS*** (n=10)

and (iv) **HS + BMMNC** (n=13). Rats were treated with either 1 ml/kg PBS or  $1 \times 10^7$  BMMNC suspended in 1 ml/kg PBS upon resuscitation. Sham-operated rats underwent identical surgical procedures but without hemorrhage or resuscitation.

**Western Blot Analysis.** Western blots were carried out as previously described [39]. Three separate experiments of western blot analysis were performed for each marker and tissues were done separately for each western blot experiment. Briefly, rat liver and lung samples were homogenized and centrifuged at 4,000 *g* for 5 min at 4°C. Supernatants were removed and centrifuged at 15,000 *g* at 4°C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer. The suspensions were centrifuged at 15,000 *g* for 20 min at 4°C. The resulting supernatants containing nuclear proteins were carefully removed, and protein content was determined using a bicinchoninic acid (BCA) protein assay following the manufacturer's directions. Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidenedifluoride (PVDF) membrane, which was then incubated with a primary antibody (rabbit anti-total GSK-3 $\beta$ , dilution 1:200; goat anti-pGSK-3 $\beta$  Ser<sup>9</sup> dilution 1:200; rabbit anti-total Akt dilution 1:1000; mouse anti-pAkt Ser<sup>473</sup> dilution 1:1000; rabbit anti-NF- $\kappa$ B p65 dilution 1:400; rabbit anti-total ERK1/2 dilution 1:2000; mouse anti-phospho ERK1/2 dilution 1:2000). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1:10000) and developed using the ECL detection system. The immunoreactive bands were visualised by autoradiography. The membranes were stripped and incubated with  $\beta$ -actin monoclonal antibody (dilution 1:5000) and subsequently with an anti-mouse antibody (dilution 1:10000) to assess gel-loading homogeneity. Densitometric

analysis of the bands was performed using Gel Pro®Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA) and expressed as relative optical density (O.D.) corrected for the corresponding beta-actin contents and normalized using the corresponding sham data to establish relative protein expression when compared to sham animals.

**Determination of Myeloperoxidase (MPO) Activity.** Lung and liver samples were homogenised in a solution containing 0.5% (w/v) hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 *g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H<sub>2</sub>O<sub>2</sub>. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxide per min at 37°C and was expressed in milliunits per gram of wet tissue.

**Materials.** Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.). All stock solutions were prepared using non-pyrogenic saline (0.9 % [w/v] NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, U.K.). Ringer's Lactate was purchased from Baxter Healthcare Ltd. Antibodies for western blot analyses were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

**Statistical Analysis.** All values described in the text and figures are expressed as mean±standard error of the mean (SEM) for *n* observations. Each data point represents biochemical measurements obtained from up to 13 separate

animals. Statistical analysis was carried out using GraphPad Prism 5.03 (GraphPad Software, San Diego, California, USA). Data without repeated measurements was assessed by one-way ANOVA followed by Dunnett's *post hoc* test. Data with repeated measurements was assessed by two-way ANOVA followed by Bonferroni's *post hoc* test. A *P* value of less than 0.05 was considered to be significant.

## COMPETING INTERESTS

The authors have declared that no competing interests exist.

## AUTHOR CONTRIBUTIONS

KKN, NSAP and CT were involved in the conception, hypotheses delineation, and design of the study; KKN, KT, NSAP, MC, EB and CT were involved in the acquisition of the data or the analysis and interpretation of such information, and KKN, KT, NSAP, MC and CT were involved in writing the article or substantial involvement in its revision prior to submission.

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## FIGURE LEGENDS

**Figure 1: Effect of BMMNC administration on the circulatory failure caused by hemorrhagic shock.** Mean arterial pressure was monitored throughout hemorrhage and resuscitation subsequent to sham-operation (Sham + PBS, n=10; Sham + BMMNC, n=4) or hemorrhagic shock (HS + PBS, n=10; HS + BMMNC, n=13). Data represent mean±SEM for n observations, \* $P<0.05$  Sham vs. HS + PBS, # $P<0.05$  HS + PBS vs. HS + BMMNC.

**Figure 2: Effect of BMMNC administration on the organ injury, dysfunction and inflammation caused by hemorrhagic shock.** Effect of BMMNC on renal function (A + B); serum urea and creatinine levels, liver injury (C + D); serum aspartate aminotransferase and alanine aminotransferase levels, and neuromuscular injury (E); serum creatine kinase levels; were measured subsequent to sham-operation (Sham + PBS, n=10; Sham + BMMNC, n=4) or hemorrhagic shock (HS + PBS, n=10; HS + BMMNC, n=13). Data represent mean±SEM for n observations, \* $P<0.05$  Sham vs. HS + PBS, # $P<0.05$  HS + PBS vs. HS + BMMNC.

**Figure 3: Effect of BMMNC on lung and liver inflammation.** Activity of myeloperoxidase in the liver (A) and lung (B) subsequent to sham-operation (Sham + PBS, n=3) or hemorrhagic shock (HS + PBS, n=3; HS + BMMNC, n=3). Data represent mean±SEM for n observations, \* $P<0.05$  Sham vs. HS + PBS, # $P<0.05$  HS + PBS vs. HS + BMMNC.

**Figure 4: Effect of BMMNC on the phosphorylation of Akt and GSK-3 $\beta$  in the livers and lungs of rats that underwent hemorrhage and resuscitation.** Phosphorylation of Ser<sup>473</sup> on Akt in the liver (A) and lung (B), Ser<sup>9</sup> on GSK-3 $\beta$  in the liver (C) and lung (D), subsequent to sham-operation (Sham + PBS, n=3) or hemorrhagic shock (HS + PBS, n=3; HS + BMMNC, n=3). Data represent mean $\pm$ SEM for n observations, \* $P$ <0.05 Sham vs. HS + PBS, # $P$ <0.05 HS + PBS vs. HS + BMMNC.

**Figure 5: Effect of BMMNC on the nuclear translocation of the p65 NF- $\kappa$ B subunit in the livers and lungs of rats that underwent hemorrhage and resuscitation.** NF- $\kappa$ B nuclear translocation in the liver (A) and lung (B) subsequent to sham-operation (Sham + PBS, n=3) or hemorrhagic shock (HS + PBS, n=3; HS + BMMNC, n=3). Data represent mean $\pm$ SEM for n observations, \* $P$ <0.05 Sham vs. HS + PBS, # $P$ <0.05 HS + PBS vs. HS + BMMNC.

**Figure 6: Effect of BMMNC on the phosphorylation of ERK1/2 MAPK in the livers and lungs of rats that underwent hemorrhage and resuscitation.** Phosphorylation of ERK1/2 MAPK in the liver (A) and lung (B), subsequent to sham-operation (Sham + PBS, n=3) or hemorrhagic shock (HS + PBS, n=3; HS + BMMNC, n=3). Black bars indicate ERK1 phosphorylation and patterned bars indicate ERK2. Data represent mean $\pm$ SEM for n observations, \* $P$ <0.05 Sham vs. HS + PBS, # $P$ <0.05 HS + PBS vs. HS + BMMNC.

**Figure 7: Effect of BMMNC on the expression of ICAM-1 in the livers and lungs of rats that underwent hemorrhage and resuscitation.** Expression of ICAM-1 in

the liver (A) and lung (B), subsequent to sham-operation (Sham + PBS, n=3) or hemorrhagic shock (HS + PBS, n=3; HS + BMMNC, n=3). Data represent mean±SEM for n observations, \* $P<0.05$  Sham vs. HS + PBS, # $P<0.05$  HS + PBS vs. HS + BMMNC.

Figure 1

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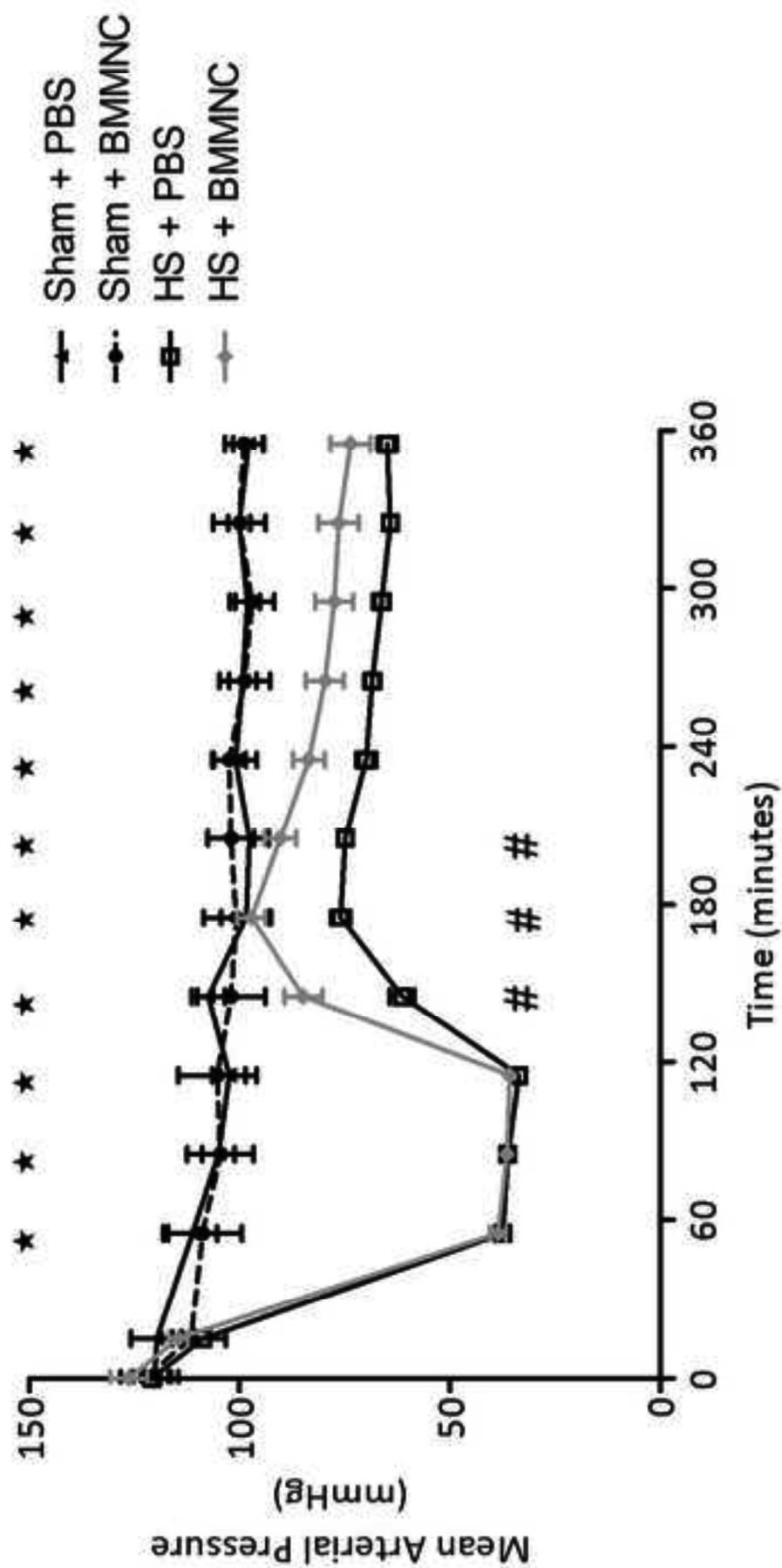


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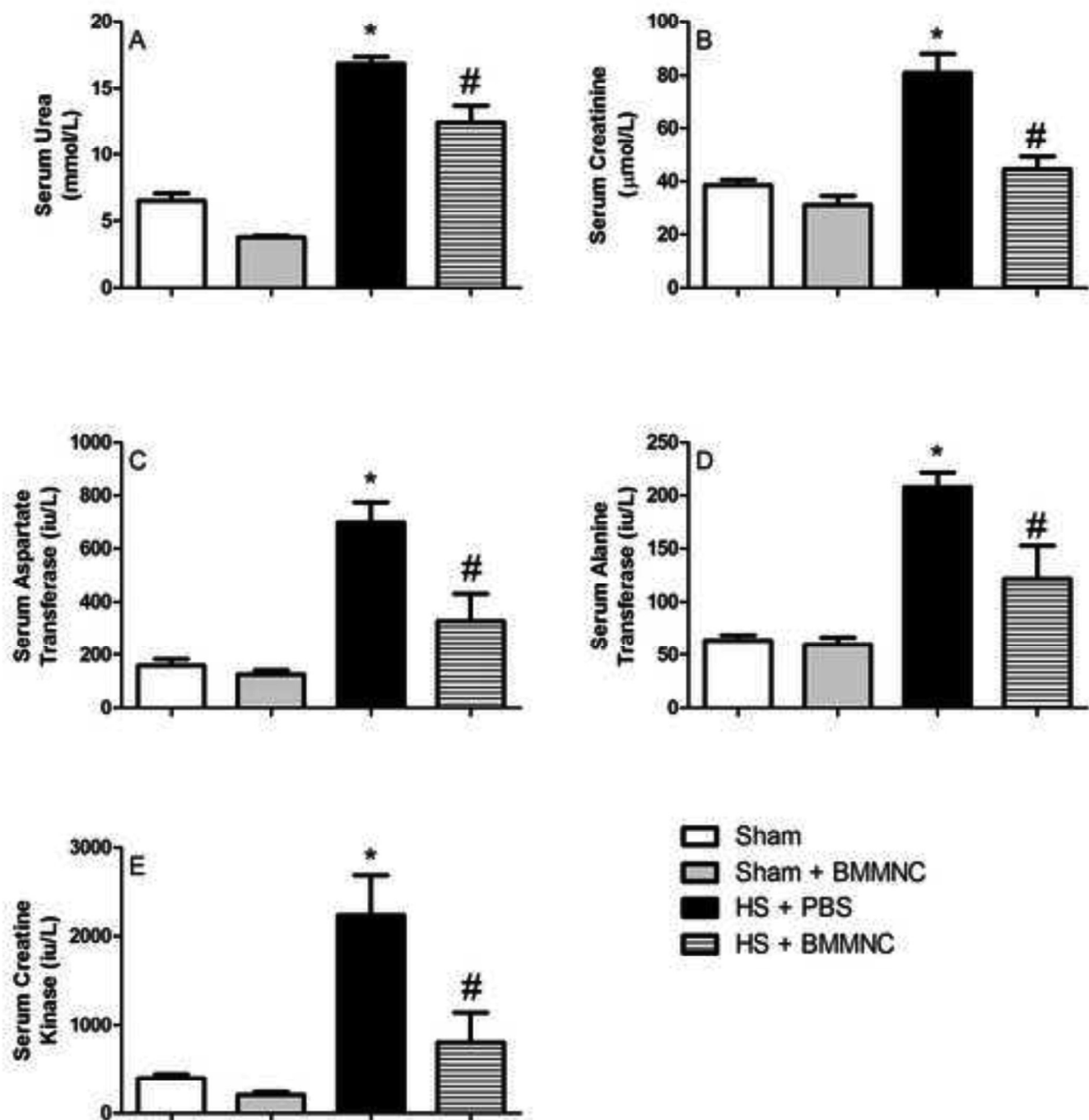


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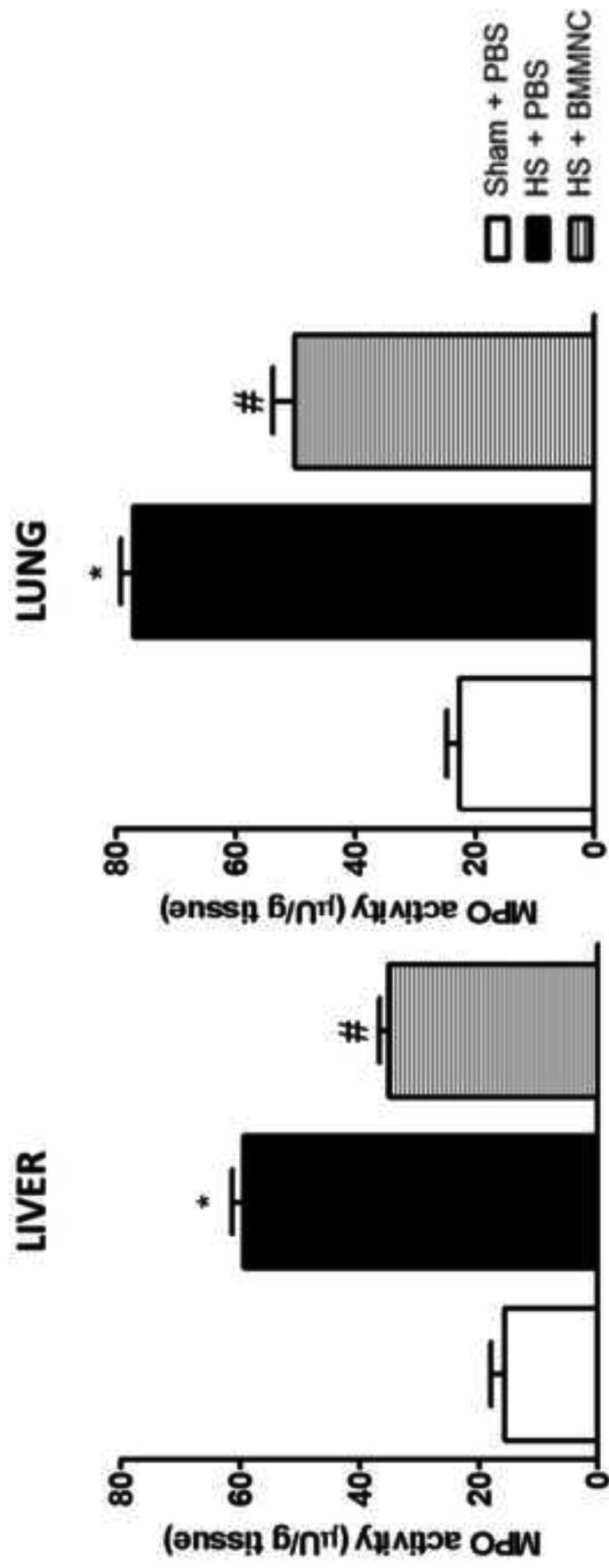


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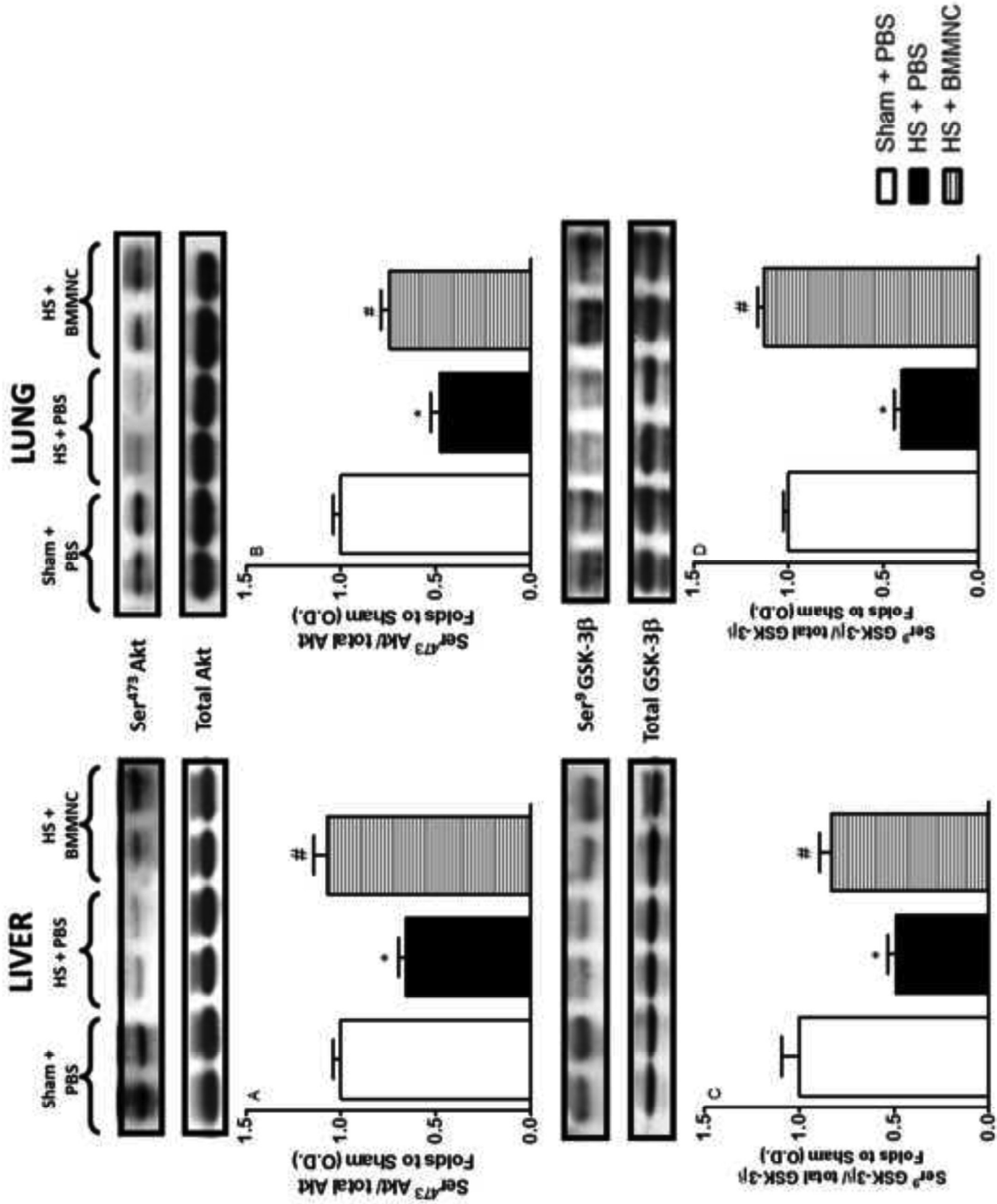


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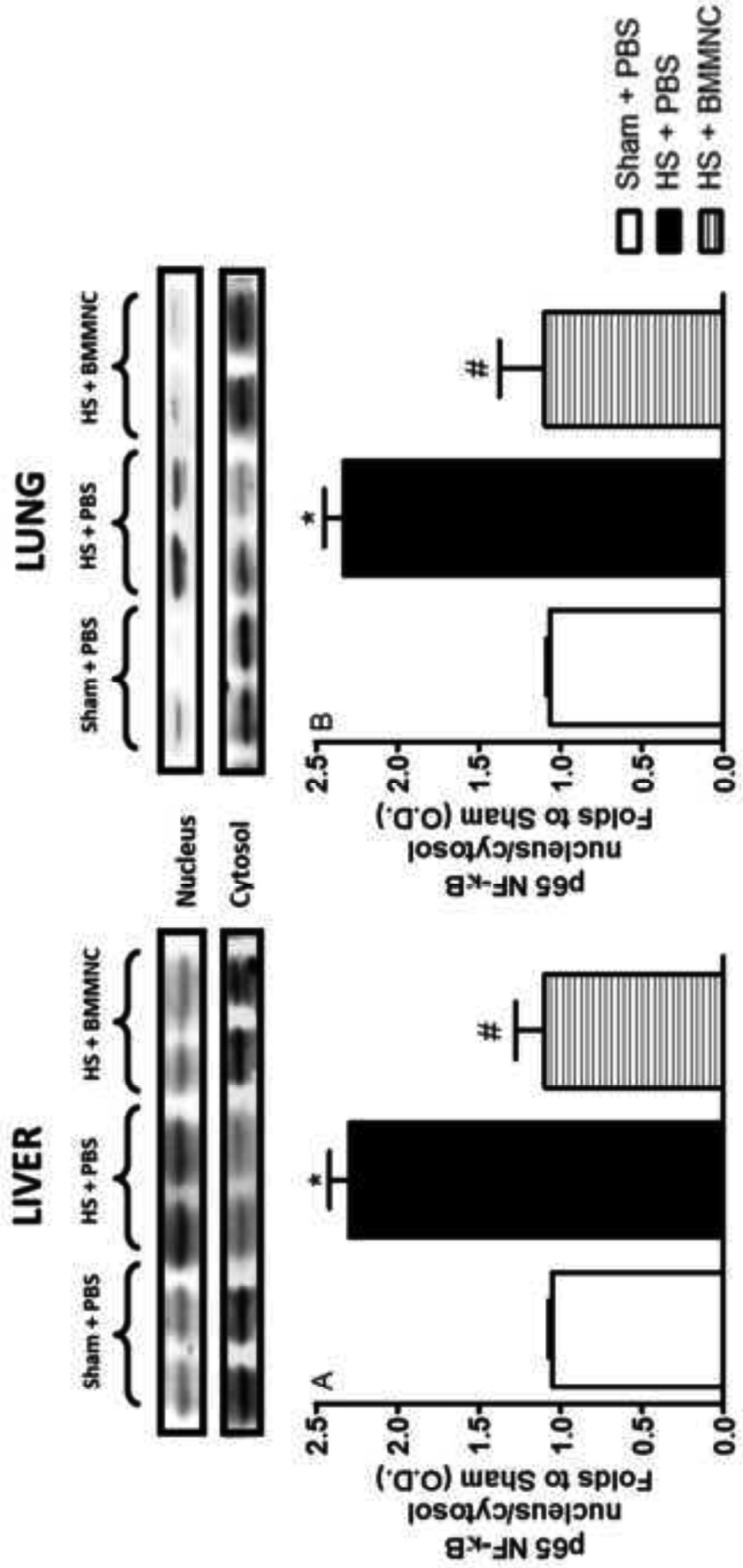




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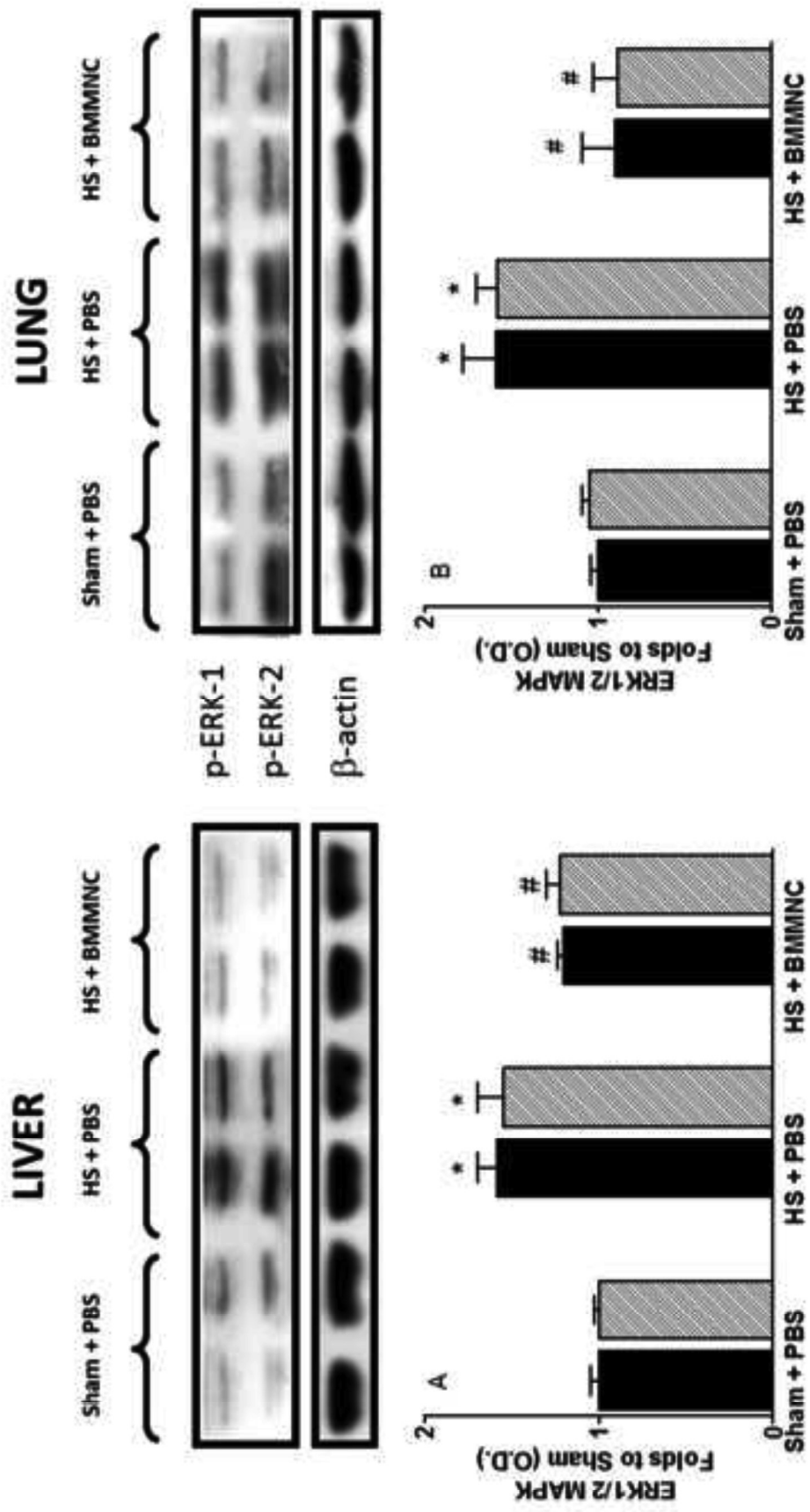


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